Identification and characterisation of a novel splice variant of synaptojanin1

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Abstract Synaptojanin1, the major constitutively active $PtdInsP_3$ 5-phosphatase activity in rat brain, is one of two closely related proteins both extensively spliced in their C-terminal proline rich domain. We describe here the discovery of a novel splice variant of synaptojanin1 which misses the major N-terminal part of the SAC1 domain. This ΔSAC-synaptojanin1 is expressed in rat brain tissue as shown by Northern and Western analysis. However, the deletion of the SAC1 domain does not alter $PtdInsP_3$ 5-phosphatase activity demonstrating that the SAC1 domain is not necessary for catalytic function.

Key words: Splice variant; Synaptojanin; Inositol; 5-Phosphatase; Lipid

1. Introduction

Inositol polyphosphate 5-phosphatases are enzymes which remove phosphates at the 5-hydroxyl position of phosphoinositides and inositol polyphosphates. Both substrates and products are implicated in signal transduction processes suggesting that these 5-phosphatases might play a role in controlling these events [1]. In particular, two 5-phosphatases have been the focus of recent investigations, SHIP and synaptojanin [2–6]. The latter has been shown to be the principal constitutively active $PtdInsP_3$ 5-phosphatase in brain tissue suggesting that it might be involved in the regulation of phosphoinositide 3-kinase dependent signalling [7]. Synaptojanin is also supposed to be part of the machinery which controls or modulates synaptic vesicle transport due to its unique tissue distribution and the subcellular localisation of binding partners [1,4,8–12]. Thus, synaptojanin seems to participate in the phosphoinositide 3-kinase dependent membrane traffic in neuronal cells [1].

To date two distinct, but highly conserved gene products termed synaptojanin1 and synaptojanin2 have been identified [4,11,12]. We have now mapped the synaptojanin1 gene to mouse chromosome X, region D (R. Woscholski and J.A. Williamson, unpublished observation). As such, it will be of interest to know whether this is reflected in any autosomal disorders. Synaptojanin2 has yet to be mapped. Both of these genes are transcribed to produce multiple processed transcripts that reflect splice sites in the C-terminal proline rich domain [4,13,14]. These forms seem to be differentially expressed in neuronal cells at different developmental stages and localised to distinct subcellular compartments [4,11,12]. One form, a particular splice form of synaptojanin1 containing a 16 amino acid insert in the proline rich domain, is the major transcript in the brain and accounts for nearly all of the constitutively active $PtdInsP_3$ 5-phosphatase [7]. We describe here the discovery of a novel splice form of synaptojanin1 characterised by a truncated N-terminal SAC domain. Expression of this variant demonstrates that the SAC domain is not necessary for catalytic function.

2. Materials and methods

2.1. Cloning of synaptojanin splice variant

Synaptojanin1 was purified from rat brain tissue and subjected to microsequencing as described [15]. Degenerate primers were then designed for a 44 aa peptide covering the core 5-phosphatase domain (K743–Y784). These primers were then used to amplify the corresponding cDNA from a rat brain library (Stratagene) by polymerase chain reaction (PCR). A PCR product sequenced and found to be identical to the sequence obtained by microsequencing of the purified synaptojanin1 [15] was then random labelled and employed in a screen using the same library. We have described elsewhere the identity of the purified synaptojanin [7]. However, in addition we isolated an N-terminal truncated synaptojanin1 missing almost three quarters of the N-terminal SAC1 domain which is referred to here as ΔSAC-synaptojanin1 (accession number AJ006855).

2.2. Northern blot and sequence analysis

Sequencing was performed on an ABI Prism Sequencer (Perkin Elmer) using the ABI Prism fluorescence labelling kit. Northern blots of different tissues were obtained from Clontech. The blots were washed in hybridisation buffer (Church's mix) at 50°C. The probes employed were either end or random labelled and employed at the indicated temperature using Church's mix. The synaptojanin1 common probe was generated by cutting out the coding sequence with *Hin*dIII and *Nhe*I (2623 bp) and was then used for random labelling, while the ΔSAC-synaptojanin1 specific probe was generated by cutting out a 165 bp fragment from the ΔSAC-synaptojanin1 clone using *Eco*RI and *Sph*I and subsequently end labelled. The blots were then visualised by autoradiography or phosphorimager scanning.

2.3. Antibodies and protein analysis

Antibodies against the insert and C-terminal region were raised and employed for Western blotting as described [7]. Rat brains were removed immediately and frozen in liquid nitrogen. Soluble and particulate fractions were obtained as described before [15]. Protein and 5-phosphatase activity were determined as previously documented [15].

3. Results and discussion

3.1. Cloning of a novel synaptojanin1 splice variant

We recently purified the constitutively active $PtdInsP_3$ 5-phosphatase from rat brain [15] which then was subjected to microsequencing. The resulting peptide sequence was found to be novel, but related to the inositol polyphosphate 5-phosphatase core sequences [7], indicating that the purified $PtdInsP_3$ 5-phosphatase has to be a novel member of the widening inositol polyphosphate 5-phosphatase family [1]. While the clon-

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GAATTCGGCACGAGGCCATGTAGTAAAACATCAGCATGTAGACGGCAGGTGTGCTAACTGTTTCCTATTTTTATGGTTATTTTGTGCCTG    90
R  I  R  H  E  A  M  .  .  N  I  S  M  .  T  A  G  V  L  T  V  S  Y  F  Y  G  Y  F  V  P
AAAATGGTGGCGGTGTAAAGTTGGAAGTGGAAGGGCATGCAGTTTTCTTCCTGTCTGTTTCAGCCAGTTTGTTTATACTTTATTCCTGGT   180
E  N  G  G  G  V  K  L  E  V  E  G  H  A  V  F  F  L  S  V  S  A  S  L  F  I  L  Y  S  W
GTAATCTAGCTGTGTAACTATCGAATTGTTACAGGTGCCAGAGTGGTACAGTTCGGACAAACTGCCTGGACTGTCTGGATAGAACAAACA   270
C  N  L  A  V  .  L  S  N  C  Y  R  C  Q  S  G  T  V  R  T  N  C  L  D  C  L  D  R  T  N
GTGTGCAGGCGTTCCTTGGCTTAGAGATGCTAGCTAAACAGCTGGAAGCTCTGGGCTTGGCTGAAAAGCCTCAGTTGGTGACTCGCTTTC   360
S  V  Q  A  F  L  G  L  E  M  L  A  K  Q  L  E  A  L  G  L  A  E  K  P  Q  L  V  T  R  F
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Fig. 1. Sequence of the 5′ ΔSAC-synaptojanin1 clone. The first 360 bp of the ΔSAC-synaptojanin1 clone are shown. The novel sequence and its translation is boxed; the underlined translated sequence represents the start of the coding sequence. The *Eco*RI site used for the creation of the library is shown in italics.

ing was in progress McPherson et al. [4] cloned a novel 5-phosphatase, called synaptojanin (now renamed synaptojanin1) which was identical in its sequence to the peptide and cDNA sequence obtained at that time. However, all clones had two base pair changes (A1904G and A2883G) and possessed a unique 16 aa splice insert at the 3′ end of the open reading frame. Thus, it seemed that the purified $PtdInsP_3$ 5-phosphatase is identical with synaptojanin1 which was then subsequently proven by immunoprecipitation experiments using specific antibodies and employment of recombinant synaptojanin1 [7]. Although all clones were identical to the described synaptojanin1 sequence, one clone seemed to contain a unique 5′ truncation (see Fig. 1). This particular clone had a stretch of about 200 bp unique sequence at the 5′ end of the clone between the critical *Eco*RI site (cloning site of the library) and the known coding synaptojanin1 sequence suggesting that this particular synaptojanin1 clone might to be a possible splice variant. This N-terminal truncated synaptojanin1 missing almost three quarters of the N-terminal SAC1 domain [4] will from now on be referred to as ΔSAC-synaptojanin1.

In order to determine if ΔSAC-synaptojanin1 is expressed in vivo a Northern blot analysis was carried out. Probes were designed either to be unique for the ΔSAC-synaptojanin1 splice variant (probe: ΔSAC-synaptojanin1 specific consisting of the first 200 bp from the 5′ non-coding region) or to be void of any unique sequence (probe: common synaptojanin1; comprising 2.6 kb of coding region). As shown in Fig. 2, the Northern blot analysis revealed that the synaptojanin1 specific probe hybridised in brain tissue with several differently sized mRNAs. A much shorter mRNA product was detected in lung tissue. However, as shown before [4,11] synaptojanin1 seems to be expressed at highest levels in brain. The synaptojanin1 common probe detected several messages in rat brain (arrows in Fig. 2) as compared to lung and heart tissue where only the shorter message was detected. The ΔSAC-synaptojanin1 specific message detected in principle only one message per tissue but of different size: the highest level in lung fol-

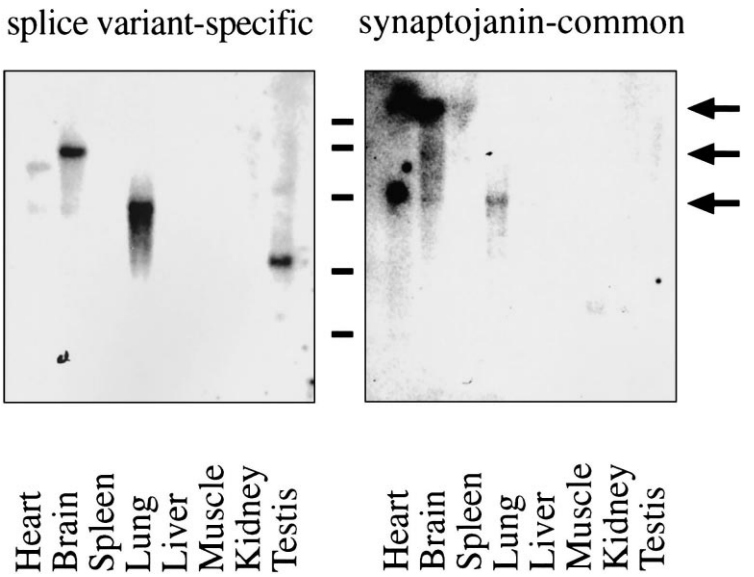

Fig. 2. Northern blot analysis of rat tissues. Probes against the unique 5′ end of the ΔSAC-synaptojanin1 splice variant (left) and the coding synaptojanin1 sequence (right) were prepared as described in Section 2. The probes were then hybridised with the rat brain tissue blot (Clontech) according to the manufacturer's instructions at 50°C. Labelled bands were visualised by autoradiography. The position of molecular weight markers (from top to bottom: 9.5, 7.5, 4.4, 2.4 and 1.35 kb) is indicated between the two autoradiographs.

lowed by brain, testis and heart. Since the synaptojanin1 specific probe and the ΔSAC-synaptojanin1 specific probe detected a similar sized message in brain (middle arrow in Fig. 2) it can be concluded that the N-terminal truncated synaptojanin1 splice variant is detectable as a 7 kb mRNA in rat brain tissue.

3.2. The ΔSAC-synaptojanin1 splice variant is expressed in rat brain tissue

Although the ΔSAC-synaptojanin1 specific cDNA probe detected a transcript in lung tissue (Fig. 2), Western blots (not shown) revealed that there is no detectable protein bearing the 16 aa insert (the antigen for the synaptojanin1 antibody) [7]. Thus, synaptojanin1and ΔSAC-synaptojanin1 with the 16 aa insert splice variation are not present at significant levels in other tissues. Since antibodies raised against the C-terminus as well as the 16 aa splice insert of synaptojanin1 did not detect protein in the other tested tissues (data not shown), detectable levels of synaptojanin1 seem to be confined to neuronal tissue. Thus, we tested whether rat brain tissue contains smaller synaptojanin forms by Western blot. As shown in Fig. 3, recombinant ΔSAC-synaptojanin1 matches one of the bands in rat brain cytosol. Taking all these observations together it is likely that ΔSAC-synaptojanin1 is expressed in brain although not at high levels compared to synaptojanin1 itself.

Since it has been argued that the SAC domain might play a role in the regulation of the activity [4,16], we investigated whether this particular splice form has altered catalytic properties. As shown in Fig. 4, the N-terminal deletion of the SAC domain did not affect the specific activity suggesting that the SAC domain is not necessary for catalytic activity. The data presented indicate that the novel splice variant ΔSAC-synaptojanin1 is expressed in rat brain tissue based on Northern and Western blot analysis. However, N-terminal deletion does not alter the intrinsic catalytic activity demonstrating that this domain is not essential for the 5-phosphatase activity. The identification of this novel ΔSAC-synaptojanin1 provides evidence that an alternative 5′ splice variation occurs in synaptojanin1. This splice product is weakly expressed as compared to the previously characterised synaptojanin1 in brain tissue.

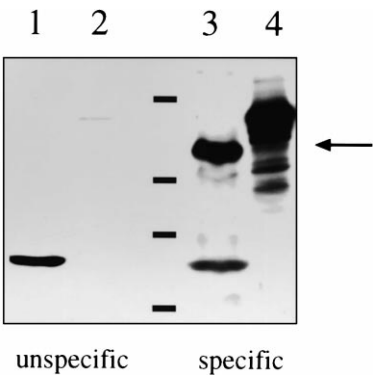

Fig. 3. Comparison of recombinant ΔSAC-synaptojanin1 and synaptojanin1 forms by Western blot. ΔSAC-synaptojanin1 was expressed in COS cells. COS cell extracts (lanes 1 and 3) and rat brain cytosol (lanes 2 and 4) were then separated by SDS polyacrylamide gel electrophoresis and blotted onto PVDF membranes. The blot was then incubated with antibodies raised against synaptojanin1 (insert). Unspecific binding was checked by incubating in the presence of competing peptide (lanes 1 and 2).

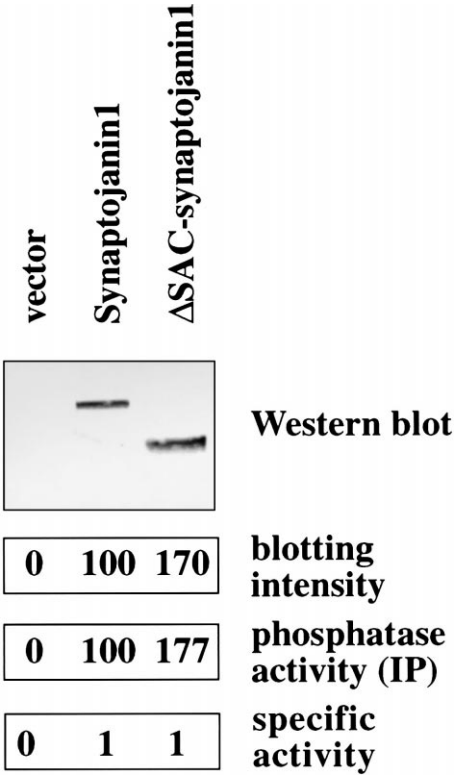

Fig. 4. Comparison of $PtdInsP_3$ 5-phosphatase activities of recombinant ΔSAC-synaptojanin1 and synaptojanin1. ΔSAC-synaptojanin1 and synaptojanin1 were expressed in COS cells and immunoprecipitated using the insert specific synaptojanin1 antibody. Expression levels were checked by Western blot (insert specific antibody) using crude extracts. Immunoprecipitates were subjected to a $PtdInsP_3$ 5-phosphatase assay. Quantification of the assay was done by phosphorimager; data are shown as relative activity. This is one of two similar experiments.

But although this ΔSAC-synaptojanin1 seems to be not abundant with respect to expression or activity, it cannot be ruled out that this splice form might be more extensively expressed in development or particular cell types. Ultimately the potential role of ΔSAC-synaptojanin1 may well be derived from elucidation of the function of the SAC domain. Evidently deletion of this domain may retrieve specific constraints on activity or localisation. This remains to be determined.

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References

[1] Woscholski, R. and Parker, P.J. (1997) Trends Biochem. Sci. 22, 427–431.
[2] De Smedt, F., Verjans, B., Mailleux, P. and Erneux, C. (1994) FEBS Lett. 347, 69–72.
[3] Liu, L., Damen, J.E., Ware, M.D. and Krystal, G. (1997) J. Biol. Chem. 272, 10998–11001.
[4] McPherson, P.S., Garcia, E.P., Slepnev, V.I., David, C., Zhang, X., Grabs, D., Sossin, W.S., Bauerfeind, R., Nemoto, Y. and De Camilli, P. (1996) Nature 379, 353–357.
[5] Giuriato, S., Payrastre, B., Drayer, L.A., Plantavid, M., Woscholski, R., Parker, P.J., Erneux, C. and Chap, H. (1997) J. Biol. Chem. 272, 26857–26863.
[6] Pesesse, X., Deleu, S., De Smedt, F., Drayer, L. and Erneux, C. (1997) Biochem. Biophys. Res. Commun. 239, 697–700.

[7] Woscholski, R., Finan, P.M., Radley, E., Totty, N.F., Sterling, A.E., Hsuan, J.J., Waterfield, M.D. and Parker, P.J. (1997) J. Biol. Chem. 272, 9625–9628.
[8] de Heuvel, E., Bell, A.W., Ramjaun, A.R., Wong, K., Sossin, W.S. and McPherson, P.S. (1997) J. Biol. Chem. 272, 8710–8716.
[9] Micheva, K.D., Kay, B.K. and McPherson, P.S. (1997) J. Biol. Chem. 272, 27239–27245.
[10] Li, J.Y., De Camilli, P. and Dahlstrom, A. (1997) Eur. J. Neurosci. 9, 1864–1874.
[11] Haffner, C. et al. (1997) FEBS Lett. 419, 175–180.
[12] Nemoto, Y., Arribas, M., Haffner, C. and DeCamilli, P. (1997) J. Biol. Chem. 272, 30817–30821.
[13] Ramjaun, A.R. and McPherson, P.S. (1996) J. Biol. Chem. 271, 24856–24861.
[14] Khvotchev, M. and Sudhof, T.C. (1998) J. Biol. Chem. 273, 2306–2311.
[15] Woscholski, R., Waterfield, M.D. and Parker, P.J. (1995) J. Biol. Chem. 270, 31001–31007.
[16] De Camilli, P., Emr, S.D., McPherson, P.S. and Novick, P. (1996) Science 271, 1533–1539.